Reduction of an *Escherichia coli* K12 Population by *Bdellovibrio bacteriovorus* Under Various In Vitro Conditions of Parasite:Host Ratio, Temperature, or pH

ABSTRACT

Intrinsic and extrinsic parameters of food products, as well as bacterial population, were evaluated for their effects on the ability of *Bdellovibrio bacteriovorus*, a bacterium parasitic upon gram-negative bacteria, to reduce an *Escherichia coli* population. High concentrations of both parasite and host were the most effective for reducing a specified *E. coli* population. *B. bacteriovorus* was able to reduce the *E. coli* count by 90% (1 log) in < 1 h at ratios of 5:1, 10:1, and 30:1 (parasite:host). Temperatures between 20 and 30°C were more conducive to bdellovibrio attack than temperatures less than 20°C. *E. coli* populations were reduced by more than 7-log values after 7 h of incubation at 30°C with parasite:host ratios of 2:1, 5:1, and 10:1. Greater than a 5-log reduction in the *E. coli* population was observed at the ratio of 30:1. *B. bacteriovorus* reduced the *E. coli* population by 1 log in approximately 24 min and 20 min at pH 7.2 and 6.8, respectively. At pH values <6.8, the activity of *B. bacteriovorus* was diminished. These results define some of the conditions where the application of *B. bacteriovorus* may aid in the reduction/elimination of some gram-negative pathogens and spoilage flora that may be present in foods.

Bdellovibrio bacteriovorus, a gram-negative, aerobic, parasitic bacterium, was first described by Stolp and Petzold (*12*). *B. bacteriovorus* is parasitic upon gram-negative bacteria such as *Escherichia coli*, *Salmonella* sp., *Shigella* sp., and *Vibrio* sp. (*10*). Bdellovibrios have been found in soil and sewage (*6*), rivers and marine environments (*9,13,17*). They alternate between a free-living, motile attack phase and an intracellular, filamentous growth phase. Bdellovibrios reproduce by first randomly encountering and then attaching to a susceptible gram-negative host bacterium. They proceed to penetrate the host outer membrane and peptidoglycan layer and lodge themselves in the periplasmic space of the host where they consume the prey cell contents and increase in length followed by septation and release of attack phase progeny by host cell lysis (*2*).

Due to the perishability of food products, there is a strong interest in extending the shelf life of foods, particularly refrigerated foods. In addition to proper temperature control, numerous methods are being tested and developed to extend the shelf life of refrigerated foods - alteration of package atmosphere, treatment of foods with organic acids, or use of bacteriocin-producing bacteria (*1,4,5,8*). A new potential method for extending the shelf life of foods and reducing the likelihood of gram-negative bacterial food-borne disease is the addition of parasitic bacteria to the food product. This study was conducted to determine some of the parameters applicable to food products which would allow effective attack by *B. bacteriovorus*, using *E. coli* K12 as a representative host (prey) organism.

METHODOLOGY

Bacterial strains

Bdellovibrio bacteriovorus 109J (Bd) and *Escherichia coli* K12 were donated by Dr. John Tudor (St. Joseph's University, Philadelphia, PA). *B. bacteriovorus* was propagated on *E. coli* K12 in dilute nutrient broth [DNB (*11*)]. *E. coli* K12 was maintained on nutrient agar (NA, Difco Laboratories, Detroit, MI).

Bdellovibrio *propagation*

E. coli K12 was grown overnight (18-24 h) in 500 ml of nutrient broth (NB; Difco) at 30°C at 260 rpm. Cells were harvested by centrifugation (5,000 x g for 20 min at 4°C) and suspended in 500 ml of DNB. Two milliliters of an overnight culture of *B. bacteriovorus* was added to 200 ml of DNB which contained between 1 x 10^8 and 2 x 10^8 *E. coli* K12 per ml. This culture was shaken overnight at 30°C at 260 rpm for 12-16 h. Bdellovibrios were harvested by centrifugation (10,000 x g for 20 min at 4°C) and suspended in 1 ml of 3 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-1 mM $CaCl_2$-0.1 mM $MgCl_2$ [HMB, pH 7.4 (*14*)].

In Vitro reduction of E. coli *K12 by* B. bacteriovorus

The effect of parasite:host ratio upon reduction of a population of *E. coli* K12 by *B. bacteriovorus* was evaluated by using *E. coli* K12 grown overnight in 500 ml of NB at 30°C at 260 rpm. Cells were harvested by centrifugation and suspended in 20 ml of HMB to a cell concentration of 1 - 2 x 10^{10} CFU/ml. The cell concentrations of *B. bacteriovorus* and *E. coli* K12 used for treatment inoculation were determined by spectrophotometer readings at 540 nm and compared to previously determined growth

curves. Spectrophotometric readings of cell concentrations for treatment inoculation were verified by plating on NA. Varying ratios (2:1 to 10^8:1) of Bd/*E. coli* K12 cell suspensions (Bdellovibrio concentration constant at 1 x 10^{10}/ml) were mixed in HMB and incubated at 30°C at 400 rpm for 7 h. Samples were withdrawn at intervals and surviving *E. coli* K12 were plated on NA with a Spiral Plater (Model D, Spiral Systems, Inc., Bethesda, MD). After incubation at 35°C for 16-18 h, *E. coli* K12 colonies were counted with a Model 500A Bacteria Colony Counter (Spiral Systems, Inc.).

The effect of temperature upon reduction of a population of *E. coli* K12 by *B. bacteriovorus* was evaluated using a ratio of 2:1 (1 x 10^{10} Bd per ml:5 x 10^9 *E. coli* K12 per ml). Cell suspensions were mixed in HMB and incubated at 30, 25, 20, 15, and 10°C at 400 rpm for 7-120 h. Samples were withdrawn at intervals, plated, and incubated on NA as described above.

A ratio of 10^5:1 (1 x 10^{10} Bd per ml:1 x 10^5 *E. coli* K12 per ml) was employed to study the effect of pH upon reduction of a population of *E. coli* K12 by *B. bacteriovorus*. Cell suspensions were mixed in HMB at pH values of 7.2, 6.8, 6.4, 6.0, 5.6, and 5.2. Suspensions were incubated at 30°C for 7 h, samples were withdrawn, plated, and incubated on NA as described above.

A different prey:host ratio was used in the pH experiment than in the temperature. A ratio of 2:1 was used in the temperature study because the literature has shown that this is the optimum condition for bdellovibrio propagation. The pH study was conducted at a ratio of 10^5:1 for two reasons. A ratio of 10^5:1 has been shown to be the cutoff level for reduction of a host population by bdellovibrio. Also, with the initial bdellovibrio population at 10^{10} per ml, an *E. coli* population of 10^5/ml is "more feasible" for food product simulation than 10^9 *E. coli* per ml (2:1 prey:host ratio).

In all of the experimental runs, each experiment was conducted in duplicate and replicated twice. In all of the figures, each data point represents the means of the experimental data.

RESULTS AND DISCUSSION

The effect of parasite:host ratio upon the effectiveness of *B. bacteriovorus* to reduce an *E. coli* population is shown in Table 1 and Fig. 1. If the bdellovibrio population was held constant and the *E. coli* level was varied, high levels of host were conducive for the destruction of *E. coli*. An increase in the parasite:host ratio (high Bd/low *E. coli*) led to an increase in the length of time to reduce the *E. coli* population by 1 log. Varon and Ziegler (*16*) have suggested that with high concentrations of bdellovibrio but low concentrations of prey, the bdellovibrio cannot find the host readily. Their only means of finding a host is by random collision. *B. bacteriovorus* was able to reduce the *E. coli* count by 1 log in < 1 h at ratios of 5:1, 10:1, and 30:1 (parasite:host) (Table 1). After 7 h of incubation at 30°C, the *E. coli* population at ratios of 2:1, 5:1, and 10:1 was reduced by more than 7-log values, whereas greater than a 5-log reduction was observed at the ratio of 30:1 (Fig. 1). The remainder of the parasite:host ratios tested yielded approximately a 2- or 3-log reduction after 7 h of incubation at 30°C (Fig. 1). *B. bacteriovorus* was found to be the most effective down to levels of 10^5 *E. coli* K12 per ml under the conditions tested. Varon and Shilo (*15*) observed that the highest percentage of bdellovibrio attached to *E. coli* occurred as the parasite:host ratio decreased from 3:1 to 1:10, and attachment was optimal at a ratio of 1 bdellovibrio to 10 *E. coli* host cells. Attachment of bdellovibrio to the host cell does not necessarily infer a decrease in the host cell population. Bdellovibrio must parasitize the host cell before the attack can be effective. *B. bacteriovorus* locates a host bacterium by random encounter and must find a host bacterium in order to survive. Bdellovibrio use a great deal of energy in their quest for a host, and if unable to find a suitable host before the exhaustion of their energy reserves, they will expire.

B. bacteriovorus more effectively reduced the bacterial population at temperatures between 20 and 30°C than below 20°C (Fig. 2). The length of time to reduce the *E. coli* population by 1-log at 30°C was 70 min. A 1-log decrease in the *E. coli* population at 25 and 20°C occurred after 161 and 192 min, respectively. Greater than a 5-log reduction in the *E. coli* population was achieved at 30°C after 6 h of incubation, while the same degree of reduction was achieved at 25 and 20°C after 24 h of incubation. At temperatures below 20°C, the efficiency of *B. bacteriovorus* was greatly reduced. The time for a 1-log decrease at 15°C was 862 min while after 120 h at 10°C, the host

TABLE 1. *The effect of parasite:host ratio upon number of* E. coli *K12 survivors.*

	Ratio of *B. bacteriovorus*/*E. coli* K12[a]											
	2:1	5:1	10:1	30:1	50:1	100:1	10^3:1	10^4:1	10^5:1	10^6:1	10^7:1	10^8:1
Time (h)	*E. coli* K12 surviors (Log CFU/ml)[b]											
0	9.87	9.46	9.14	8.60	8.57	8.48	7.26	6.32	5.80	4.77	4.38	4.54
0.25	9.53	9.38	8.61	8.15	8.66	7.88	7.21	6.14	5.40	4.62	4.11	4.04
0.5	9.60	8.69	7.72	7.56	8.43	8.08	6.94	5.97	5.62	4.61	4.28	4.29
1	9.42	8.23	6.89	7.37	8.12	7.82	6.62	5.44	5.29	4.42	3.72	3.85
2	9.23	7.10	5.78	6.29	7.59	7.46	5.92	4.75	3.83	4.27	3.37	3.52
3	8.25	6.37	4.24	5.20	7.21	6.88	6.02	4.45	3.44	3.62	2.93	2.75
4	5.37	5.23	3.09	4.10	6.76	6.64	5.69	4.15	2.96	3.19	2.59	2.59
5	5.08	3.34	2.26	3.01	6.45	6.31	5.26	3.84	2.75	3.47	1.78	1.74
6	3.92	3.24	1.76	2.69	6.02	6.12	4.84	4.04	2.63	2.91	1.79	1.87
7	2.48	2.13	1.83	2.78	5.51	5.67	4.86	3.65	2.66	2.93	1.83	1.87

[a] *Bdellovibrio* population constant at 1 x 10^{10}/ml.

[b] Mean log values of experiments conducted in duplicate and replicated twice.

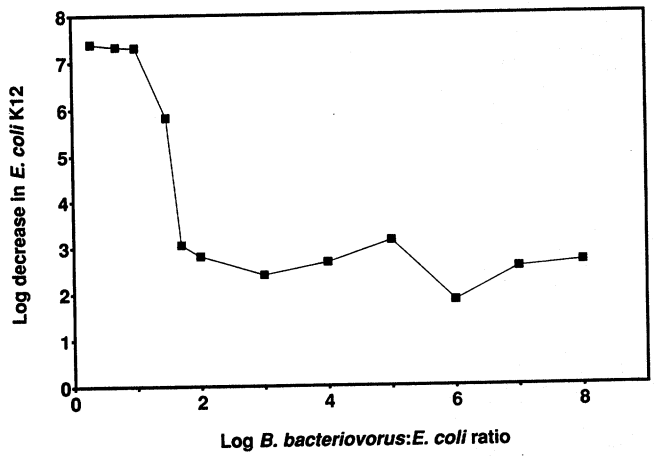

Figure 1. *The effect of parasite:host ratio upon the effectiveness of* B. bacteriovorus *to reduce an* E. coli *population after 7 h at 30°C.* B. bacteriovorus *population at 1 x 10^{10}/ml.*

bacterial population had decreased by only 0.16 logs. Lambina et al. (*7*) observed that under conditions of sewage purification, the maximal parasite:host interaction was achieved at 26°C and the minimal 1 at 19°C. Final *E. coli* populations of sewage purified at 26 or 19°C and transferred to a precipitation tank were 8 x 10^2 CFU/ml and 4 x 10^3 CFU/ml, respectively. Varon and Shilo (*15*) found that the highest degree of attachment by *B. bacteriovorus* to *E. coli* was achieved at 30-35°C. They observed that bdellovibrio attachment was slow at 20°C and negligible at less than 15°C. Filip et al. (*3*) observed an "appreciable" number of plaques (10-20 per petri dish) by both a wild type and groundwater strain of *B. bacteriovorus* at a minimum of 18°C. The optimum temperature for plaque formation was found between 26 and 30°C. These results show that *B. bacteriovorus* is more active at temperatures >20°C; however, their research did not address the effect of temperature upon the ability of bdellovibrio to reduce/eliminate a specified *E. coli* population.

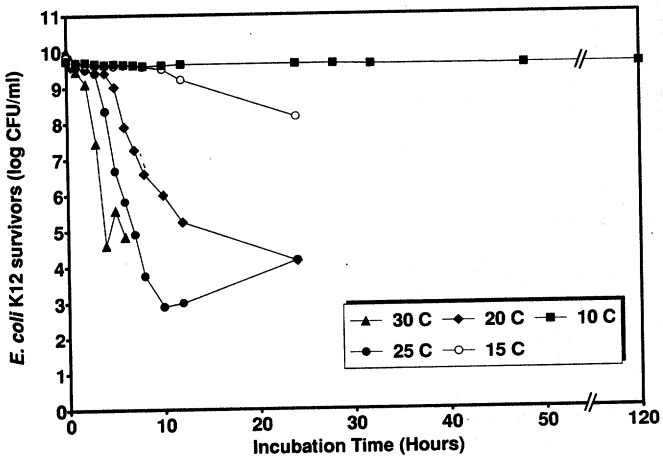

Figure 2. *The effect of temperature upon the ability of* B. bacteriovorus *to reduce an* E. coli *population. Initial* E. coli *population at 10^9/ml.*

B. bacteriovorus was found to work best at pH values near neutrality (Fig. 3). *B. bacteriovorus* reduced the *E. coli* population by 1-log in approximately 24 and 20 min at pH 7.2 and 6.8, respectively. After 7 h of incubation at 30°C, the *E. coli* population at pH 7.2 and 6.8 decreased by approximately 2 and 3 logs, respectively. The remainder of the pH values tested yielded < 1.5-log reduction after 7 h at 30°C. At pH values of 6.4, 6.0, 5.6, and 5.2, the time for *B. bacteriovorus* to reduce the *E. coli* K12 population by 1-log was approximately 337, 96, 118, and 151 min, respectively. Filip et al. (*3*) observed that bdellovibrio did not form plaques at pH values less than 6.0; however, numerous plaques were found at pH values between 7 and 9. Varon and Shilo (*15*) found that *B. bacteriovorus* lost their motility at pH 5.0 and were therefore unable to attach to host cells.

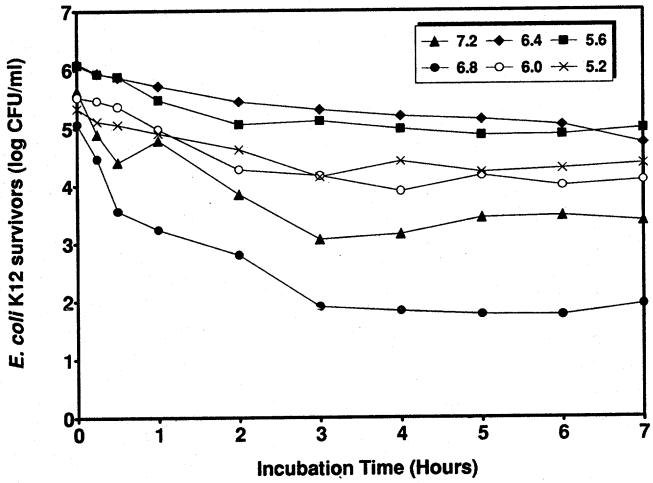

Figure 3. *The effect of pH upon the ability of* B. bacteriovorus *to reduce an* E. coli *population. Initial* E. coli *population at 10^5/ml.*

Previous bdellovibrio research has dealt with the effect of various conditions upon the degree of attachment of bdellovibrio to host cells or the number of plaques formed. We have examined the effect of some conditions applicable to food products for their effect upon the ability of *B. bacteriovorus* to reduce a specified *E. coli* K12 population. The ability of *B. bacteriovorus* to parasitize at temperatures between 20 and 30°C makes it ideal for use in foods which are stored at ambient temperature or have been subjected to abuse temperatures. Many food products have pH values near neutrality which would provide a suitable environment for *B. bacteriovorus*. *B. bacteriovorus* may be applied to the surface of foods in a buffered spray or dip form. Foods where they may be used are whole fruits and poultry, fish or seafood prior to packaging. Research is being conducted on the ability of *B. bacteriovorus* to attack a variety of gram-negative foodborne spoilage and pathogenic bacteria as well as the usefulness of *B. bacteriovorus* as a surface application on chicken skin and other food and food equipment surfaces.

ACKNOWLEDGMENTS

The authors would like to thank Dr. John Tudor, St. Joseph's University, Philadelphia, Pennsylvania, for his assistance with this research.

REFERENCES

1. Anderson, M. E., and R. T. Marshall. 1989. Interaction of concentration and temperature of acetic acid solution on reduction of various species of microorganisms on beef surfaces. J. Food Prot. 52:312-315.